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TECHNICAL MANUSCRIPT 344

NEUROLOGICAL AND PHYSIOLOGICAL RESPONSES OF THE PRIMATE TO ANTHRAX TOXIN

James A. Vick
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FEBRUARY 1967

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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Process Development Division
AGENT DEVELOPMENT AND ENGINEERING LABORATORY

Project 1C522301A059

February 1967

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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ABSTRACT

Anthrax toxin depressed the cerebral cortical electrical activity of both anesthetized and nonanesthetized monkeys and anesthetized chimpanzees. Recordings on a physiograph recorder revealed changes in electrocardiogram and hypoxic hypertension that progressed with the degree of intoxication to final cardiovascular collapse. Changes in cortical electrical activity were either partial or complete, and in some cases cortical activity was depressed in cyclic patterns that appeared independent of other observed physiological changes. Subcortical changes in electrical activity occurred simultaneously with the surface cortical changes. The protective antigen component of the toxin alone caused the initial changes in surface cortical activity. Those animals that died showed a respiratory failure that appeared to be of central nervous system origin involving the respiratory center of the brain. Survival with no changes in physiological or cortical electrical activity occurred in monkeys pretreated (30 min) or post-treated (up to 8 hours) with specific antiserum.

I. INTRODUCTION

DeMoulin[1] observed that the central and peripheral nervous systems were involved and damaged in animals dying of anthrax. Aside from scattered references bearing on possible nervous system involvement due to anthrax, little attention was paid to this subject until the review of Lincoln et al.[2] who summarized the experimental observations that indicate central nervous system (CNS) involvement after challenge with either spores or sterile anthrax toxin. These authors proposed a model of action for anthrax based on the direct effect of toxin on the CNS. They considered that most of the observations on this disease, such as numerous cellular and chemical changes in the blood and sudden death in humans and animals, could most readily be explained by involvement of the CNS.

The gross symptoms in animals and many of the pathophysiological changes following the administration of toxins such as botulinum, E. coli endotoxin, and snake venom appear remarkably similar to changes observed following challenge with anthrax toxin. Vick, Ciuchta, and Polley[3] and Polley et al.[4] have shown depression of cortical electrical activity in experimental animals challenged with botulinum toxin or snake venoms. They also have shown that the respiratory failure associated with botulinum intoxication is characterized by interference with transmission over the neuromuscular junction of the diaphragm. The techniques used by Vick and colleagues were applied in this study to further elucidate the possible action of anthrax toxin on the central nervous system and to correlate this change with those occurring in other physiological parameters. This study furnishes information on the mechanism of the "sudden death" phenomenon commonly observed with anthrax.

II. MATERIALS AND METHODS

Anthrax toxin was prepared by the method of Haines, Klein, and Lincoln,[5] concentrated by freeze drying, and resuspended so that 25 ml contained 10,000 rat units of toxin, an amount selected to cause death between 24 and 36 hours. The characteristics of the crude toxin are given in Table 1. The components, protective antigen (PA), lethal factor (LF), and edema factor (EF) were separated by the method of Beall, Taylor and Thorne.[6] The eluates containing EF were combined and passed through a diethylaminoeth l (DEAE) cellulose column to remove traces of LF. The components were dialyzed using LKB filters (LKB 6300 A ultra filter, LKB Produkter AB, Stockholm, Sweden) and concentrated by freeze drying.[7]

TABLE 1. CHARACTERISTICS OF THE COMPLETE TOXIN MOLECULE

Lot	Protein,[a] mg/ml	Dry Weight,[b] mg/ml	Rat Lethality,[c] units/ml	Guinea Pig[d] Edema Titer	Ouchterlony[e] Titers LF	PA
1	1.24	15.8	32	1:50	1:8	1:32
2	1.46	17.4	36	1:8	1:8	1:64
3	1.07	13.6	37	1:16	1:8	1:16
4	1.36	12.9	21	1:32	1:8	1:32
5	1.41	14.0	28	1:4	1:8	1:64

a. Protein was determined spectrophtometrically by the method of Waddell.[8]
b. Dry weight of uninoculated medium components is 7.5 mg/ml including 2.2 mg/ml of glucose.
c. Rat lethality was determined by the method of Haines et al.[5]
d. Guinea pig skin edema was titered by the method of Thorne et al.[9]
e. The Ouchterlony procedure of Thorne and Belton[10] was modified with an azocarmine stain.[7] Two lines corresponding to protective and lethal factor were observed. Edema factor could not be detected.

Lethal preparations (10,000 rat units) were prepared by recombining PA and LF in a ratio of 4:1. EF was combined in a 3:1 ratio with PA. The characteristics of the toxin components are given in Table 2.

Specific antiserum, prepared in horses against the Sterne strain of Bacillus anthracis by the Sclavo method, was purchased from Institute Serateropico Tuscano, Sienna, Italy. Each milliliter of antiserum neutralized approximately 7,000 rat units of toxin produced in vitro.

Twenty rhesus monkeys (Macaca mulatta) weighing between 3.9 and 5.4 kg and two chimpanzees (Pan troglodytes) weighing 22 and 27 kg were used in this study. Simultaneous and continuous recordings of cerebral cortical electrical activity (EEG), blood pressure, electrocardiograms (EKG), heart, and respiratory rate were taken on unanesthetized animals, except for the three animals on which phrenic nerve impulses were recorded. The EEG was recorded with permanent electrodes surgically implanted 21 days prior to challenge on the dura of the brain by standard stereotaxic techniques. EEG recordings were made with a Model 5 Grass polygraph.*

* Grass Instruments, Quincy, Mass.

Heart rate, EKG (precortical lead), and respiratory rate were monitored with a pair of needle-tip electrodes placed subcutaneously in either side of the chest. Blood pressure was monitored with a Statham strain gauge and an E and M Physiograph recorder.* The Statham strain gauge was connected to a fine polyethylene catheter filled with heparinized saline inserted in the femoral artery and advanced into the distal abdominal aorta. Venous catheters were placed in the saphenous vein to facilitate injections.

TABLE 2. CHARACTERISTICS OF TOXIN COMPONENTS

Component	Protein,[a] mg/ml	Dry Weight, mg/ml	Rat Lethality,[b] units/ml	Guinea Pig[c] Edema Titer	Ouchterlony[d] Titer
EF	0.393	16.7[e]	0	1:40	1:2
	0.039	12.1	0	1:10	1:1
PA	0.750	10.8[f]	0	0	1:128
	0.985	12.4	0	0	1:32
	1.220	13.3	0	0	1:32
	1.263	13.8	0	0	1:16
LF	0.157	15.2[e]	70	0	1:32
	1.090	28.7	7	0	1:8
	0.620	29.9	94	0	1:16
	0.422	29.5	142	0	1:16

a. Protein was determined spectrophotometrically by the method of Waddell [8]
b. Rat lethality was determined by the method of Haines et al.[6]
c. Guinea pig skin edema was titered by the method of Thorne et al.[9]
d. The Ouchterlony procedure of Thorne and Belton[10] was modified with an azocarmine stain.[7] A single line corresponding to each component was observed.
e. In some experiments 0.15 M carbonate buffer containing 14.3 mg/ml of solids was used as eluting fluid; in later experiments 0.3 M carbonate buffer was used.
f. Dry weight of uninoculated medium was 7.5 mg/ml including 2.2 grams per liter of glucose.

* E and M Instrument Co., Houston, Texas.

Nerve impulse traffic over the central end of the cut left phrenic nerve was followed in three monkeys maintained at a surgical stage of anesthesia with Brevital* (methohexital sodium). The peripheral end of the cut phrenic nerve and the diaphragm were also stimulated periodically with a Grass Model S4 stimulator.

The 20 monkeys in this study were divided at random into three groups: Group I consisted of five monkeys given 10,000 units of toxin and observed until death. Three of these five monkeys were studied for phrenic nerve activity. Group II consisted of five monkeys pretreated with 70 ml of antiserum 1 to 2 hours prior to injection of 10,000 units of toxin. Group III consisted of ten monkeys challenged with 10,000 units of toxin, then initially treated at 30 minutes to 14 hours post-injection with 70 ml of antisera. Subsequent injections of 25 ml of antiserum were made at 4 and 8 hours following initial antiserum injection.

The survivors of Groups II and III, listed in Table 3, were re-challenged 10 to 52 days after the first toxin challenge with a second injection of 10,000 units of anthrax toxin.

An additional group of four rhesus monkeys and two chimpanzees were anesthetized with Nembutal 30 mg/kg (sodium pentobarbital) to study the effect of anthrax toxin on subcortical areas of the brain. Depth electrodes were placed in the amygdala, hippocampus, lateral gyniculate, and reticular formation by standard stereotaxic procedures previously described in detail.[11] Continuous recordings of electrical activity were taken from these areas of the brain as well as from the cerebral cortex. In addition, blood pressure, electrocardiogram, heart rate, and respiratory rate were recorded at 15-minute intervals before and after the injection of toxin. Each monkey received 10,000 units toxin intravenously and each chimpanzee 100,000 units toxin intravenously. At death serial sections were made of the brain tissues to confirm the anatomical placement of each depth electrode.

* Eli Lilly and Co., Indianapolis, Indiana.

TABLE 3. EEG ACTIVITY AND TIME TO DEATH OF TREATED AND UNTREATED MONKEYS[a]

Monkey	Antiserum, Therapy, hr	EEG Depression at Indicated Time, min[b]	Survival Time, hr
	Group I: Untreated		
1[c]	None	C at 7	34
2[c]	None	C at 4	26
3	None	P at 7	28
4[c]	None	C at 5.5	31
5	None	P at 8	32
	Group II: Pretreated at times indicated		
6	-1	None	S[d]
7	-1½	None	S
8	-1½	None	S
9	-1	None	S
10	-2	None	S
	Group III: Post-treated at times indicated		
11	+½	P at 5	S
12	+1	C at 7	S
13	+2	P at 6	S
14	+4	P at 5	S
15	+8	P at 4	S
16	+8	C at 5	60
17	+10	P at 7	52
18	+11	P at 7	48
19	+12	P at 6	44
20	+14	P at 7	46

a. The response data refer to rhesus monkeys of 3.9 to 5.4 kg challenged with 10,000 units of anthrax toxin. Group I shows effects on untreated animals; Group II and Group III show effects after treatment with specific antiserum. Terminal apnea was observed in all animals that died.

b. C indicates complete, P partial loss of EEG activity at indicated time.

c. Anesthetized, phrenic nerve monitored.

d. Survived.

III. RESULTS

Actual tracings representative of cortical electrical activity and physiological changes in normal unchallenged and toxin-challenged animals are shown in Figure 1. In the anesthetized monkey (Fig. 1A) the heart rate is shown as an integrated mean, the wider the line the slower the heart beat. The EKG was normal with the exception of a slightly elevated T wave, a condition not unusual in anesthetized animals. The heart rate was 140 and blood pressure approximately 130/85. Right and left cortical EEG were distinct as characteristic of normal brain activity. Phrenic discharges occurred at the inspiratory phase of respiration. Intermittent photostimulation showed the monkey capable of responding to a light flash administered through the cornea of the eye as evidenced by increased activity of the EEG.

Table 3, Group I, presents the effects of toxin on EEG and survival time of the five untreated monkeys challenged with 10,000 units of anthrax toxin. All animals that died showed terminal apnea followed by anoxic cardiovascular collapse at 26 to 34 hours. Immediately following the administration of 10,000 units of toxin a transitory fall in the blood pressure (20 to 30 mm of mercury) occurred that quickly returned to normal. At 50 seconds, intermittent photostimulation produced some minor following that was erratic, showing from slight to increased activity of the EEG.

At 5 to 8 minutes postchallenge, 5 of 15 animals went into a coma; the remainder appeared semiconscious and depressed. The coma was characterized by an electrically silent EEG, the semiconscious state by increased amplitude of EEG activity (Fig. 1B). The length and depth of depression were related. The other physiological parameters measured at this time appeared unaffected, except that respiration rate decreased. The diaphragm and other muscles of respiration remained synchronous, with discharges over the phrenic nerve corresponding to the inspiratory phase of respiration. In those animals tested, response to light flash during this period of time appeared markedly depressed.

At one hour postchallenge (Fig. 1C) the animal appeared normal and was able to eat and drink. EEG and blood pressure returned to normal. The EKG was affected, however, and showed a prolongation of the PR interval with a slight elevation of the QRS segment and a definite depression of the ST wave. This change, along with a slowing of heart beat, indicated that cardiac irregularities had developed. Respiration was irregular, the phrenic nerve discharged not once but about three times per inspiration, as recorded on the physiograph. This phenomenon was observed in only one of the three animals with a cut phrenic nerve. This irregularity is interpreted as an attempt to increase respiration. Intermittent photostimulation elicited a normal response to light.

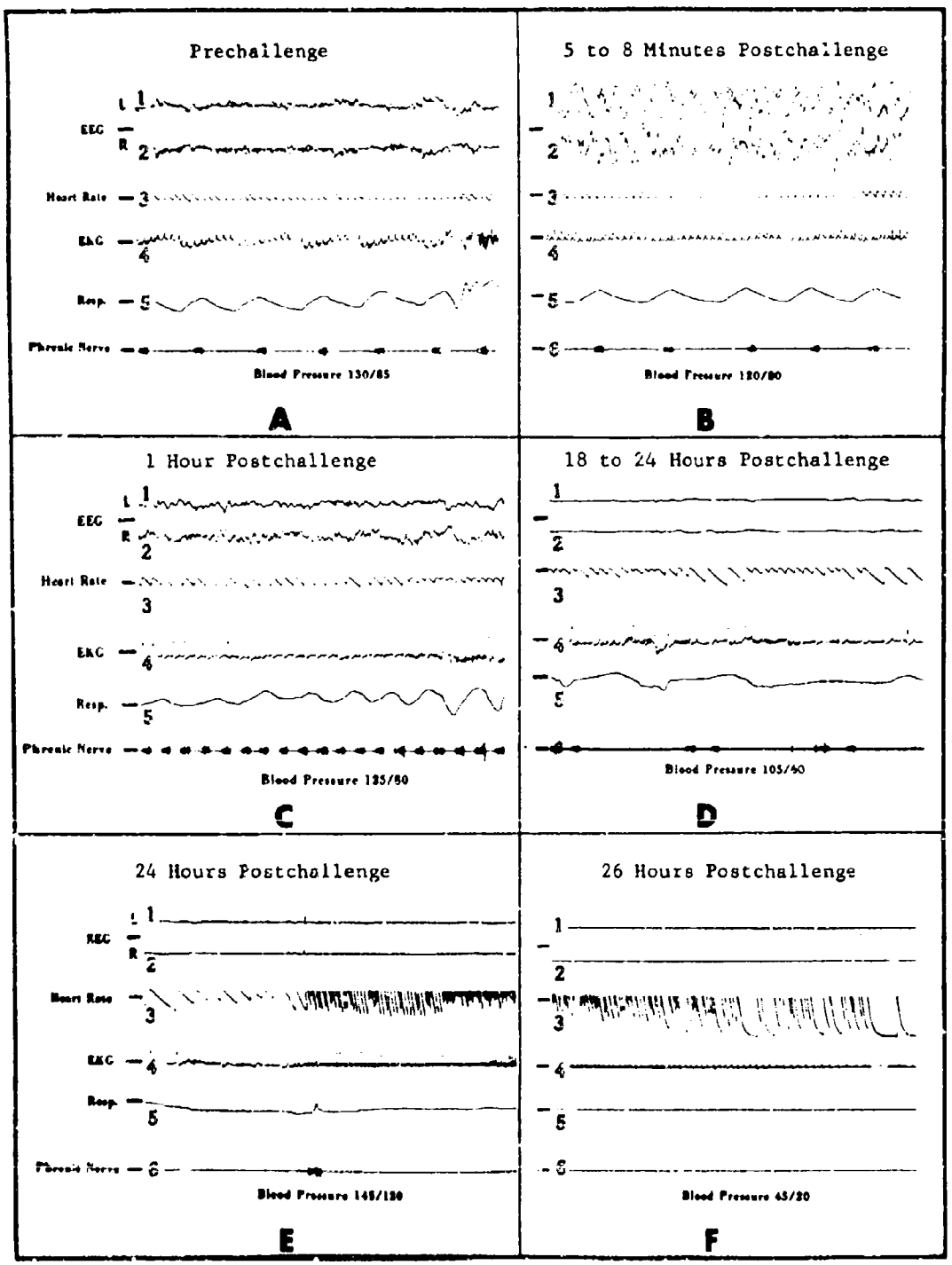

Figure 1. EEG, Heart Rate, EKG, Respiration and Phrenic Nerve Discharges in the Anesthetized Monkey after Challenge with Anthrax Toxin.

In three of the five untreated animals a cyclic pattern of cortical activity and depression occurred at intervals that varied from 5 to 8 minutes to 18 hours after injections. These responses could appear and disappear up to three times. The cyclic pattern did not appear related to the depth of the initial depression or to the time of death. The cyclic phenomenon has also been observed in monkeys challenged with botulinum toxin[4] but not those challenged with snake venom or endotoxin.[3]

At 18 to 24 hours postchallenge (Fig. 1D) a complete electrical silence in EEG was experienced. Blood pressure dropped to 105/60 mm Hg. Cardiac difficulties were evident as heart rate slowed and the EKG became grossly abnormal, indicating early myocardial ischemia. Respiration was abnormal and irregular. The phrenic nerve discharged in pairs, usually with some response from the diaphragm.

At 24 hours postchallenge (Fig. 1E) severe hypotension and respiratory difficulties were experienced. There was a typical anoxic rise in blood pressure with EKG showing progressive myocardial ischemia. Heart rate showed irregularities due to the anoxia. The diaphragm responded to the irregular phrenic discharges.

At 26 hours postchallenge (Fig. 1F) respiration ceased, followed by anoxic myocardial failure. Discharges of the phrenic nerve ceased as respiration ceased. At death, stimulation of the peripheral end of the cut phrenic nerve elicited a hyper-reactive response in the diaphragm. The response of the diaphragm indicated no block of neuromuscular transmission with anthrax toxin such as has been observed with snake venom and botulinum toxin. It appeared, rather, that the brain is depressed and no longer capable of initiating an electrical discharge. Forced ventilation was attempted with two animals, which delayed their deaths for 2 or 3 hours; death followed respiratory failure.

The two chimpanzees receiving whole toxin showed neurological and physiological effects identical in all respects to those previously described in the monkey. Gross and microscopic examination of tissues at time of death revealed complete absence of pathological lesions.

Table 3, Group II, presents results from animals that were pretreated with specific antiserum prior to challenge with anthrax toxin. In none of the pretreated monkeys were any changes observed. All animals survived. It is apparent that pretreatment with antiserum either neutralized the toxin in vivo or prevented its adsorption onto active sites.

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In order to determine how long animals could remain untreated and survive, animals were administered antiserum 30 minutes to 14 hours after challenge. Results are shown in Table 3, Group III. All animals of this group underwent the initial change in EEG and other parameters previously described showing either a partial or complete loss of EEG. One of two animals treated at 8 hours and all that were treated later died. The animals that recovered showed early signs of respiratory difficulties, then stabilized and made a complete recovery. The EEG rapidly returned to normal and all physiological parameters became normal. Those animals that died (antiserum administered after 8 hours) developed terminal apnea in spite of the amount of antiserum administered even though their times to death were markedly extended.

Table 4 includes those animals that survived initial challenge from Groups II and III of Table 3. At 10 to 52 days after initial challenge and treatment, the monkeys were again challenged with 10,000 units of toxin. Those challenged within 10 to 23 days were unaffected. However, at 30 days the animals lost resistance, which we assume to be due to passive immunity. The toxin now produced death, with an extended survival time. The three animals challenged at 30, 47, and 52 days showed the typical physiological changes and characteristics of anthrax toxicity except that the changes appeared milder. There was no evidence of active immunity being developed.

TABLE 4. RE-CHALLENGE OF SURVIVORS OF GROUPS II AND III, TABLE 3

Monkey	Days after Initial Challenge	EEG Depression at Indicated Time, min	Survival Time, hr
11	10	None	S[a]
12	14	None	S
13	18	None	S
14	18	None	S
15	20	None	S
6	21	None	S
7	23	None	S
8	30	P at 5[b]	96
9	47	P at 5	104
10	52	P at 5	84

a. Survived.
b. P indicates partial loss of EEG activity at indicated time.

Recordings of right and left cortical and six subcortical areas (Fig. 2) in the monkey and chimpanzee were comparable. Figure 2 shows the effect of a lethal injection of anthrax toxin on a chimpanzee that is considered typical of the group. Prior to challenge, all animals exhibited electrical activity characteristic of the primate under a light stage of barbituate anesthesia. Within 30 to 90 seconds after toxin was administered abrupt changes in cortical and subcortical activity occurred. There was an initial slowing of high frequency waves, followed by complete or almost complete cessation of all electrical activity (isoelectric trace) at 5 minutes. Some return of activity was noted at approximately 2 hours with no further change until just prior to death (30 hours) at which time all activity ceased. In both monkeys and chimpanzees, a simultaneous cessation of cortical and subcortical activity always preceded the respiratory difficulties and ultimate apnea. As with the animals described in Figure 1, cardiovascular failure followed respiratory arrest. The four monkeys expired at an average of 31 hours (range 27 to 36 hours) and the two chimpanzees at 59 and 83 hours postchallenge.

The administration of PA produced an initial effect much like that observed following challenge with whole toxin (Fig. 3). Within 30 to 60 seconds a marked decrease in cortical electrical activity was noted followed in some animals by complete electrical silence at approximately 3 to 5 minutes. This change was accompanied by a slight transient drop in arterial blood pressure and increase in heart rate. At 2 to 4 hours, cortical activity increased, reaching control levels at 8 hours post-injection. No further change in physiological parameters was observed following challenge with component PA. None of the animals in this group died.

When LF was administered 30 minutes after the PA component, the cortical electrical activity was once again depressed and remained depressed until death at 28 and 34 hours for the two animals of this treatment. No significant change in physiological parameters was noted until onset of acute respiratory failure resulting in death.

In contrast with PA component, the intravenous injection of LF produced no change in cortical electrical activity or in the physiological parameters monitored (Fig. 4). On administration of PA (30 minutes after LF) both monkeys showed a marked decrease in cortical activity that persisted until death. Terminal tracings show the characteristic isoelectric cortical trace, complete cessation of respiration, and progressive failure in electrocardiographic activity. Death occurred at 24 and 31 hours, respectively.

Intravenous administration of EF produced no change on any of the parameters monitored. The following controls were used in this study: (i) toxin inactivated by boiling for 30 minutes; (ii) toxin neutralized by antisera, ratio 1:3 toxin to antiserum; (iii) antisera alone; (iv) uninoculated medium processed as was the toxin; (v) nonlethal lot of toxin (inactivated during freeze-drying); (vi) phosphate buffer, 0.1 M; (vii) carbonate buffer, 0.08 M.

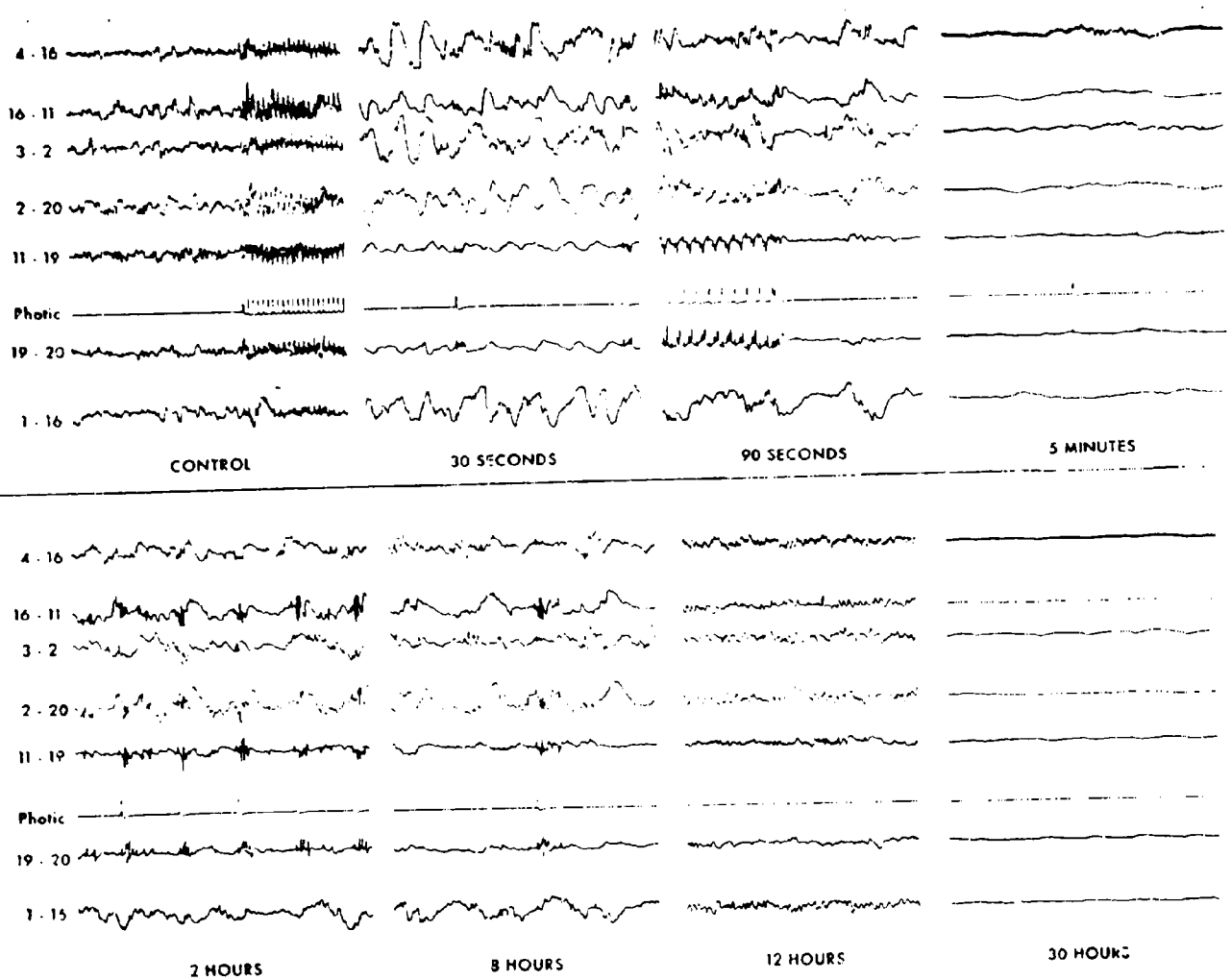

Figure 2. Effect of Lethal Injection of Anthrax Toxin on Cortical and Subcortical Activity of the Chimpanzee.

Montage: 4-16 - Right Cortex
16-11 - Amgydala
3-2 - Left Cortex
2-20 - Visual Area
11-19 - Hippocampus
Photic - Photic (Light Flash)
19-20 - Lateral Gyniculate
1-16 - Reticular Formation

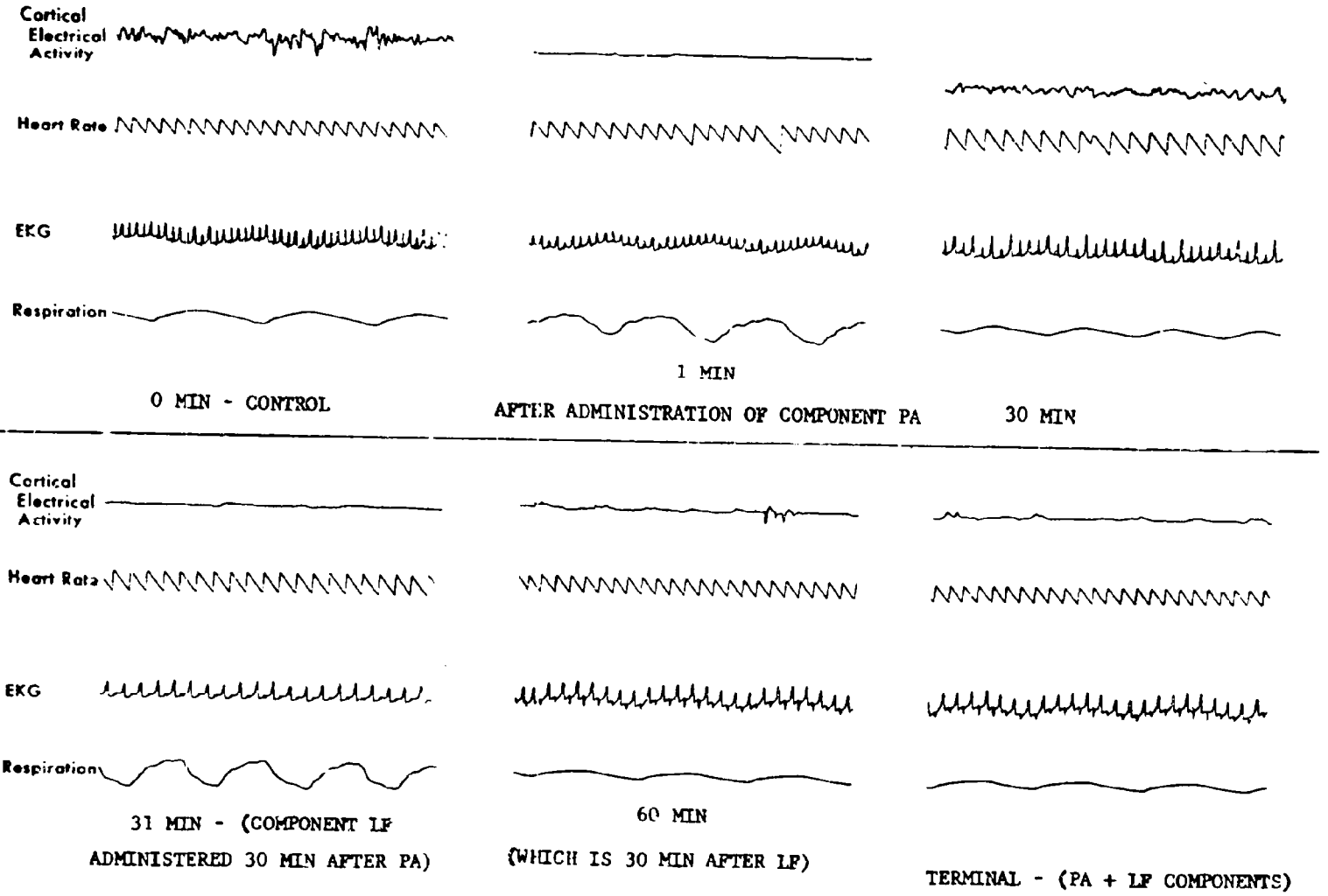

Figure 3. Response of the Monkey to the PA Component of Anthrax Toxin Given 30 Minutes Before Component LF.

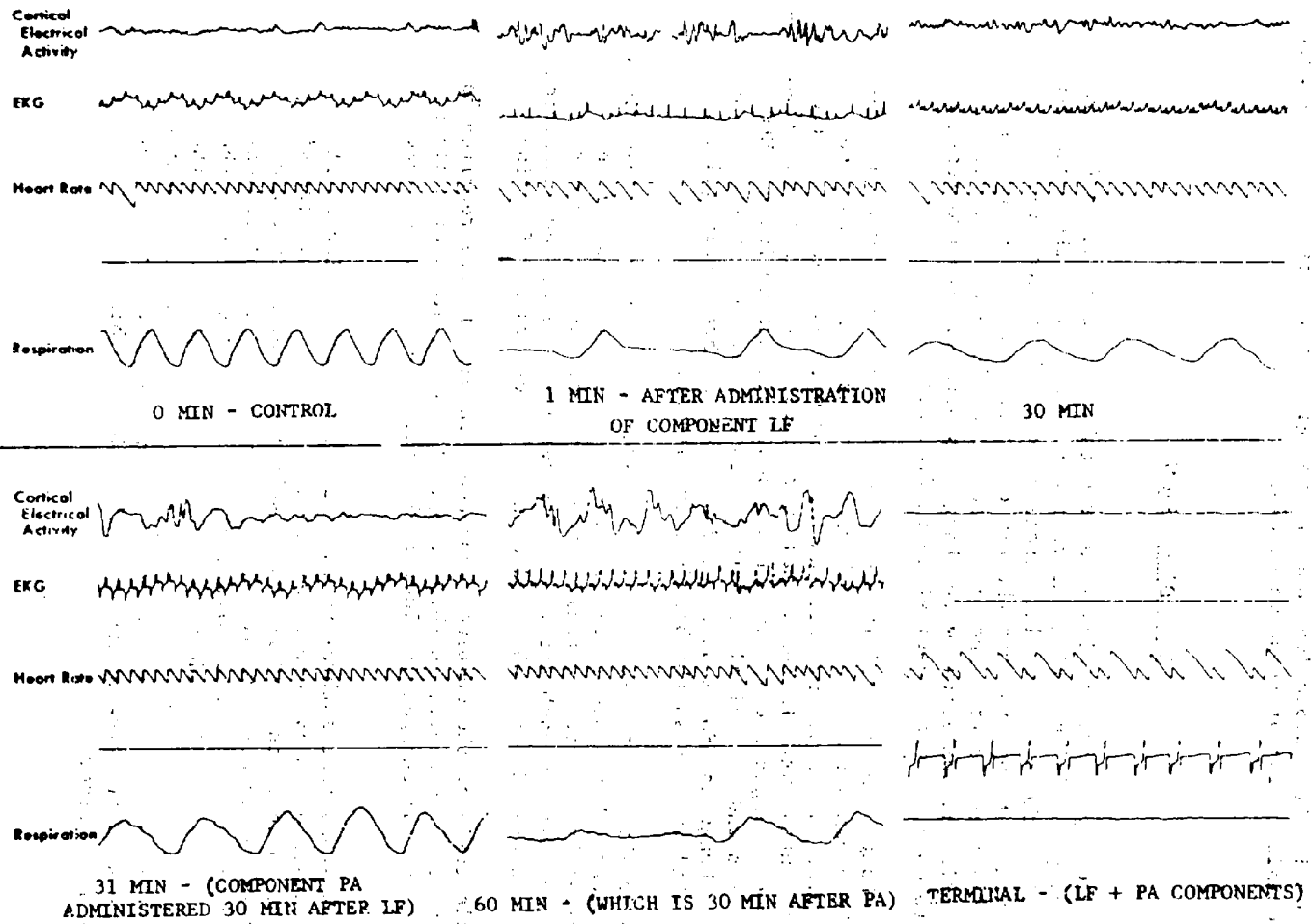

Figure 4. Response of the Monkey to the LF Component of Anthrax Toxin Given 30 Minutes Before Component PA.

No changes in the neurological and physiological parameters were observed following administration of these materials.

IV. DISCUSSION

Death caused by anthrax toxins is attributable to respiratory failure of CNS origin, probably involving the respiratory center of the brain and not peripheral at the neuromuscular junction as has been shown with botulinum toxin. It appears that the EKG changes, anoxic hypertension, and tachycardia that occurred and progressed to cardiovascular collapse were nonspecific and compatible with changes associated with progressive anoxia due to CNS-initiated respiratory difficulties.

Initial changes in EEG activity observed after challenge with anthrax toxin have been demonstrated also with snake venoms.[3] No initial EEG changes occurred upon challenge with anthrax toxin in animals pretreated with specific antiserum. If the animals were treated with antiserum after the initial EEG changes and within 8 hours postchallenge no further EEG changes occurred and all animals survived. Therefore, this initial as well as the later change in EEG is associated with the anthrax toxin and may suggest its ability to pass the blood-brain barrier.

Studies in which depth recordings were made following lethal injections of toxin indicate that subcortical, as well as cortical, areas of the brain are directly affected by toxin. Depression of activity appeared to occur simultaneously in each area. This change in cortical and subcortical activity always preceded the observed changes in respiratory function.

The whole toxin or the combined components PA plus LF are required to produce lethality. However, in this study, PA, when administered alone, produced the initial changes in electrical cortical activity observed with whole toxin.

This work shows that anthrax toxin or a lethal combination of its components causes death by respiratory failure of CNS origin. The site of action of the toxin in the CNS is most likely the respiratory center itself because the intact corneal reflexes (photostimulation) indicate little damage in the functional organization of the brain. Thus, the model proposed by Lincoln et al.[2] adequately describes the intoxication that occurs in this disease, and it is highly applicable to the treatment of anthrax. The reversal of the described syndrome by antiserum accounts for the success in treating pulmonary anthrax by a combination of antibiotics and antiserum.[1,2]

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13 ABSTRACT

Anthrax toxin depressed the cerebral cortical electrical activity of both anesthetized and nonanesthetized monkeys and anesthetized chimpanzees. Recordings on a physiograph recorder revealed changes in electrocardiogram and hypoxic hypertension that progressed with the degree of intoxication to final cardiovascular collapse. Changes in cortical electrical activity were either partial or complete, and in some cases cortical activity was depressed in cyclic patterns that appeared independent of other observed physiological changes. Subcortical changes in electrical activity occurred simultaneously with the surface cortical changes. The protective antigen component of the toxin alone caused the initial changes in surface cortical activity. Those animals that died showed a respiratory failure that appeared to be of central nervous system origin involving the respiratory center of the brain. Survival with no changes in physiological or cortical electrical activity occurred in monkeys pretreated (30 min) or posttreated (up to 8 hours) with specific antiserum.

14. Key Words

*Anthrax	Chimpanzees	Symptoms
*Toxins	Rhesus monkeys	Cardiovascular systems
*Central nervous system	Antiserums	Electroencephalography
*Physiology	Electrocardiography	Respiration
	Hypertension	Components

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